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The Red Foot of a Lepidopleurid Chiton: Evidence for Tissue Hemoglobins

by

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Abstract. Shallow-water specimens of *Leptochiton rugatus,* a member of an ancient, mostly deep-water, suborder of chitons (Lepidopleurina), have a striking red coloration of the foot and soft tissues. The possibility that the red color of various tissues of *L. rugatus* is caused by a circulating or noncirculating respiratory heme protein was investigated. A hemoglobin was identified in tissue from foot, mantle wall, gills, and surrounding the mouth. No heme protein was found in circulating hemolymph; rather a hemocyanin is likely present. It is hypothesized that the presence of this tissue hemoglobin might somehow facilitate O_2 transport in the shallow, hypoxic habitat of this animal.

INTRODUCTION

Among the diverse chiton fauna on the shores of western North America, a single species of the suborder (or order) Lepidopleurina Thiele, 1910, *Leptochiton rugatus* (Carpenter in Pilsbry, 1892), is occasionally encountered in fair numbers living in shallow water, usually on the underside of large submerged rocks at low tide. Members of the Lepidopleurina, referred to collectively here as "lepidopleurid" chitons, are generally considered the most primitive of our living chitons based on their general lack of well developed and slitted insertion plates (usually in all eight valves), their gills which are few in number and placed posteriorly, and their unspecialized tegmentum and girdle features (Kaas & Van Belle, 1985). Fossil data also support this conclusion. Over 50 fossil lepidopleurid species are known from the Paleozoic and Mesozoic eras (Van Belle, 1981) and, of these, one is possibly a *Leptochiton, L. deshayesi* (Terquem, 1852) reported from the lower Jurassic period of the Mesozoic. In contrast, species assigned to other extant families are unknown from the Paleozoic and number fewer than 10 species from the Mesozoic (Van Belle, 1981; Hoare & Smith, 1984).

As in other groups with some ancient members, the primitive lepidopleurids seem to have persisted primarily in deep waters and have been collected from depths exceeding 7000 m (review by Ferreira, 1979). The absence of lepidopleurids in exposed intertidal habitats may reflect a fundamentally different functional arrangement of gills. Based on observations of actively respiring animals, Yonge (1939) argued that the posterior gill arrangement in the lepidopleurid species *Leptochiton asellus* was less efficient for aerial respiration than the more anterior gill placement of three modern chiton species he examined. Although Yonge based his conclusions on relatively few species, his generalities concerning the morphological and habitat differences between lepidopleurids and modern chitons are probably valid, judging from reviews of gill arrangements and collection depths of more than 200 chiton species by Eernisse (1984, 1985) and Kaas & Van Belle (1985, 1986). Yonge proposed that the innovation in gill arrangement by modern chitons led to the replacement of lepidopleurids in the intertidal. As discussed by Eernisse

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(1984), it is equally plausible that lepidopleurids never were able to colonize intertidal habitats, given their posterior gill placement. It is interesting, therefore, that *L. rugatus* can occur in shallow depths as well as in deeper waters (specimens identified as *L. rugatus* have been dredged from a depth of about 458 m). If most lepidopleurids are to some extent limited to deeper water by their gill morphology, the shallow habitat of *L. rugatus* may indicate a special adaptation to shallow water conditions.

It was with the unusual shallow-water habitat in mind that we noted the distinctive red coloration of the foot, gills, and other soft parts on the underside of *Leptochiton rugatus*. As far as we know, this condition is peculiar to *L. rugatus*, and may be peculiar to only shallow-water specimens of *L. rugatus* (R. N. Clark, personal communication). It is also our experience that *L. rugatus* tends to occur in locally dense populations on the bottoms of large rocks submerged in oxygen-poor mud. This study investigates the possibility that the red coloration of the soft parts of *L. rugatus* is caused by a circulating or noncirculating respiratory heme protein which might somehow facilitate respiration in shallow, oxygen-poor habitats.

MATERIALS AND METHODS

Leptochiton rugatus was collected intertidally in May 1986 on the western shores of San Juan Island, Washington, U.S.A. (48°03′N, 123°05′W), and animals were identified according to FERREIRA (1979). The animals studied measured about 6.5–8.0 mm in length.

Ten animals were dissected. An incision was made in the mantle cavity near the gills and a very small amount of hemolymph was collected from each animal. The foot and surrounding mantle tissue were then removed and homogenized in cold phosphate buffer (0.5 M NaCl, 0.05 M sodium phosphate buffer, pH 7.5) in a scintered glass micro-homogenizer. Radular muscles were extracted in the same manner. Animal hardparts were flattened and preserved in 70% ethanol as voucher specimens (DJE coll.). Homogenates and blood samples were centrifuged at 13,000 g for 2 min and supernatants were used in the following studies.

Samples were electrophoresed on a 7.5% polyacrylamide slab gel, pH 8.9, in the absence of denaturants or reducing agents. Samples were also treated with sodium dodecyl sulfate (SDS) and dithiothreitol and electrophoresed on a 12.5% polyacrylamide SDS slab gel (for hemoglobin) or a 4% polyacrylamide SDS slab gel (for hemocyanin) (RYAN *et al.*, 1985).

The extract of foot tissue was examined spectrophotometrically using a dual beam Gibson spectrophotometer. The foot tissue extract was also chromatographed on a calibrated column (1.7 × 24 cm) of Sephadex G-75 superfine in equilibrium with the extraction buffer.

RESULTS

When *Leptochiton rugatus* was bled, the hemolymph did not appear red nor did the red color of the animal's tissues become lighter. When the foot was removed from the animal, the foot tissue retained its red color.

Figure 1

SDS PAGE of *Leptochiton rugatus* hemolymph, 4% polyacrylamide. A, *Helix aspersa* hemocyanin; B, *Leptochiton rugatus* hemolymph; C, *Katharina tunicata* hemocyanin; D, *Octopus dofleini* hemocyanin; E, calibrants (myosin, B-galactosidase, phosphorylase B).

Examination by SDS gel electrophoresis of the very small sample of hemolymph obtained from 10 animals showed no hemoglobin-like subunits. Rather, a strongly staining protein that electrophoresed to the same position as the hemocyanin subunit of *Katharina tunicata* was observed (RYAN *et al.*, 1985) (Figure 1).

The extract of tissues of foot, gills, and surrounding mantle shows absorption maxima of 414,540, and 570 nm, similar to other myoglobins and hemoglobins. The α peak is shifted slightly to the violet and is lower in absorbance than the β peak; an absorbance ratio of α/β peaks of about 0.85 suggests that the protein is susceptible to oxidation and that some met-hemoglobin is present. Attempts to chemically deoxygenate the protein with sodium dithionite resulted in a typical loss of α and β peaks and the appearance of a smooth peak at about 555 nm. However, the Soret maximum was shifted to 422–423 nm and not to 432 nm as seen for other myoglobins and hemoglobins (LEMBERG & LEGGE, 1949). The most likely interpretation of these data is that either some of the hemoglobin was not deoxygenated, some remained in the oxidized met-state (or as some other derivative) or both.

Chromatography of the sample on a column of Sephadex G-75 superfine showed a broad peak with a molecular

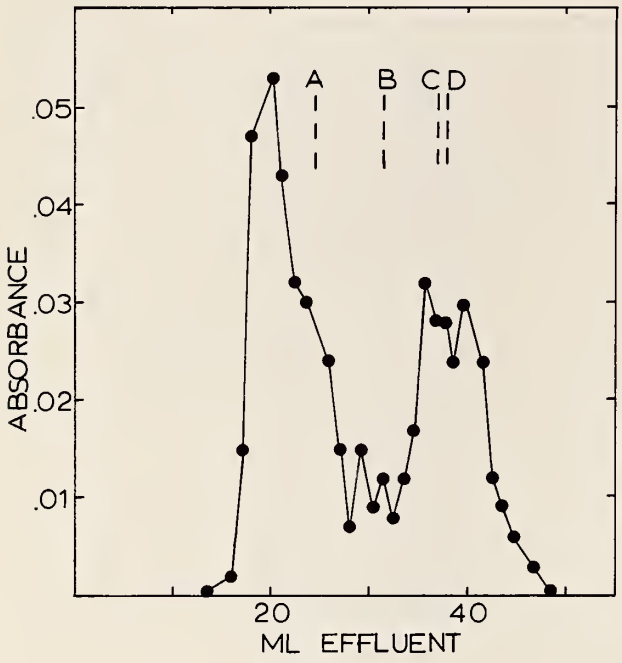

Figure 2

Chromatography of *Leptochiton rugatus* foot and mantle tissue extract on Sephadex G-75 superfine. Column buffer, 0.05 M sodium phosphate, pH 7.5, 0.5 M in NaCl. Calibrants: A, bovine serum albumen; B, *Katharina tunicata* dimeric myoglobin; C, sperm whale myoglobin; D, *Katharina tunicata* monomeric myoglobin. Absorbance at 416 nm.

weight slightly less than that of sperm whale myoglobin (Figure 2) and similar to radular muscle myoglobin of *Katharina tunicata* (Terwilliger & Read, 1970). Absorbance in the void volume was due to turbidity. SDS polyacrylamide gel electrophoresis of the foot and mantle tissue homogenate indicated a strong protein staining band with a molecular weight of about 15–16,000 (Figure 3D), similar to radular myoglobin of *K. tunicata* under the same gel conditions (Figure 3B). The radular muscles of *Leptochiton rugatus* are bright red, with an intensity similar to the redness of other chiton radular muscles (Terwilliger & Read, 1970). A prominent protein band from the radular muscles of *L. rugatus* (Figure 3C) migrated to the same distance upon SDS gel electrophoresis as myoglobins of *K. tunicata* (Figure 3B). Identification of the 15–16,000 molecular weight proteins of both foot and radular tissues as hemoglobin subunits was verified by cross-electrophoresing on SDS the red bands obtained by electrophoresis on a pH 8.9 gel in the absence of denaturants and reducing agents. Owing to very little tissue in both *L. rugatus* foot and radular muscle preparations and difficulty in collecting animals, more detailed studies were not undertaken.

DISCUSSION

Hemoglobins and myoglobins are widespread in mollusks, where they are found as circulating intra- and extracellular proteins and in tissues such as buccal muscles, heart, stomach, and nerves (Read, 1966; Terwilliger & Terwilliger, 1985). There have been no reports of hemoglobin or myoglobin in the Polyplacophora (chitons) except for myoglobins found in the bright red buccal musculature, where both monomeric and dimeric myoglobins are present (Terwilliger & Read, 1970). Furthermore, only hemocyanin has been reported as a circulating respiratory protein in chitons (Manwell, 1960; Van Holde & Miller, 1982; Ryan *et al.*, 1985). We find that *Leptochiton rugatus* resembles other chitons in having myoglobin in its radular muscles and having hemocyanin as its circulating respiratory protein. However, this is the first report of a more widespread presence of hemoglobin in other tissues of the chiton. We base our conclusion that the red color is due to a hemoglobin-like protein on (1) the protein's apparent molecular weight of 15–16,000 by gel chromatography and SDS gel electrophoresis and (2) its hemoglobin-like spectral absorption maxima which change upon deoxygenation with dithionite. Not enough hemoglobin was available for O_2-binding experiments. Experience with other tissue hemoglobins, however, suggests that because the protein is monomeric and not easily deoxygenated with dithionite, it likely has a high oxygen affinity. We are referring to this protein as a hemoglobin rather than a myoglobin because we do not know whether it is found in

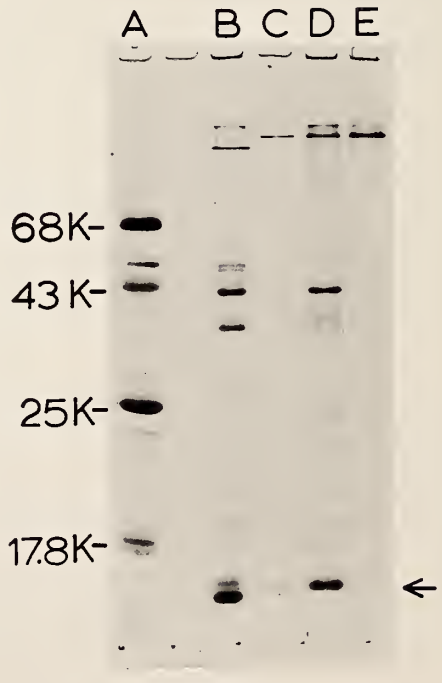

Figure 3

SDS PAGE of *Leptochiton rugatus* hemoglobin-containing tissues, 12.5% polyacrylamide. A, calibrants; B, *K. tunicata* radular muscle; C, *L. rugatus* radular muscle; D, *L. rugatus* foot and mantle tissue; E, *L. rugatus* hemolymph. Arrow indicates the position of tissue hemoglobin.

muscle fibers in all tissues in which it is present. The unusual shallow-water habitat for these chitons, under rocks that are submerged in mud, may point to a reason for the hemoglobin-rich tissue, in contrast to the presumed hemoglobin-poor tissue of lepidopleurids that occupy deep water. The presence of a high-affinity hemoglobin in *L. rugatus* might facilitate diffusion of oxygen to respiring tissues of this organism under hypoxic conditions.

Whatever the connection is between the previously mentioned posterior placement and fewer numbers of gill pairs typical of lepidopleurid chitons and their normal deep-water habitat, it remains a striking observation that, in contrast to modern chitons, there are few reported collections of lepidopleurid chitons from substrates exposed at low tide. This suggests to us that lepidopleurid chitons depend on aquatic, rather than aerial, respiration. Yet *Leptochiton rugatus* is found in a shallow-water habitat that appears oxygen poor; in fact, no modern chitons or other mollusks were observed on the same substrates heavily populated by *L. rugatus*. Moreover, at least two other lepidopleurid species have been reported from similar habitats. Iredale & Hull (1929) report *Terenochiton subtropicalis* (=*Leptochiton norfolcensis* [Hedley & Hull, 1912]) from the Sunday Islands, Kemadec Group (also known from New Zealand), collected living on the underside of embedded dirty stones below low water mark. Iredale & Hull (1929) also report collecting 60 to 80 specimens of the Australian species *Leptochiton badius* (Hedley & Hull, 1909) in one afternoon from under deeply buried stones between tide marks. A prediction consistent with our hypothesis, that the presence of tissue hemoglobins is related to the unusual habitat of *L. rugatus,* would be that the foot and other tissues of *L. subtropicalis* and *L. badius,* like those of *L. rugatus,* should be red as well.

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